Personalized Medicine in Acute Myeloid Leukemia:

Efficacy of a Novel Combination of Epigenetic Modifiers and a Tyrosine Kinase Inhibitor in Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous blood cancer from which many patients die due to ineffective or toxic treatments; thus, there is a great need to develop more effective and personalized treatment options. Many AML patient samples display abnormal epigenetic regulation, and the Mixed Lineage Leukemia (*MLL*) gene, encoding for a histone H3 lysine 4 methyltransferase, is frequently mutated in AML. The partial tandem duplication (MLL^{PTD}) is found in ~5% of cytogenetically normal AML and correlates with a poor prognosis, especially when co-present with other mutations like the internal tandem duplication (ITD) of FMS-Like Tyrosine Kinase ($FLT3^{ITD}$, a receptor tyrosine kinase). In order to better test novel therapies in a preclinical model, we developed a murine model of spontaneous AML with a double knock-in of Mll^{PTD} and $Flt3^{ITD}$ (referred to as Mll^{PTD},$Flt3^{ITD}$) with 100% penetrance and a median survival 50-60 weeks. Since MLL^{PTD} associates with DNA hypermethylation in human AML patients, we analyzed the DNA methylome in our Mll^{PTD},$Flt3^{ITD}$ model and found an increase in the global DNA methylation index in leukemic mouse bone marrow compared to non-leukemic controls similar to the hypermethylation seen in human MLL^{PTD} leukemia. Using a transplant model of our Mll^{PTD},$Flt3^{ITD}$ mouse leukemia, a combination of epigenetic modifiers [Decitabine (5AD), a DNA methyltransferase inhibitor, and AR42, a novel histone deacetylase inhibitor] effectively targeted this AML *in vivo*, reducing leukemic burden, increasing tumor suppressor expression, and increasing survival. However, the mice still succumbed to leukemia. Since our mouse also has a $Flt3^{ITD}$ mutation, we tested AC220 (a selective FLT3 inhibitor) in our mouse model. AC220 was shown to be a toxic single agent in our Mll^{PTD},$Flt3^{ITD}$ mouse model at doses required to produce killing of leukemic blasts. Since we saw toxicity with AC220 as a single agent, we asked whether combining epigenetic modifiers with AC220 would increase

efficacy and allow for a less toxic dose. Preliminary *ex vivo* data in Mll^{PTD},$Flt3^{ITD}$ leukemic blasts demonstrated that a combination of epigenetic modifiers with AC220 reduced proliferation. Ultimately, our data suggests a combination therapy of epigenetic modifiers and a FLT3 inhibitor may effectively combat AML and provide a more "personalized" therapy for human patients with these mutations.

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Chapter 1: Introduction

Acute Myeloid Leukemia:

Acute myeloid leukemia (AML) is a type of blood cancer from which most patients die due to ineffective or toxic treatment. In order to achieve better patient outcomes, personalized treatment regimens for cancer patients may improve quality of life and/or survival. Personalized medicine requires the development of drugs that target specific molecular defects that are present in a particular patient's disease. AML is an important model for personalized medicine. Current therapies available are not very effective, and patients frequently relapse. In addition, most patients are >60 years old and have difficulty tolerating the harsh toxicities of generalized chemotherapy and bone marrow transplants. The development of more specific or personalized therapies may better target leukemic cells and avoid some of the toxicity associated with current treatment options. Personalized treatments for other cancers have shown promise in human patients such as targeting the abnormal tyrosine kinase resulting from the Philadelphia chromosome (BCR-ABL) in CML. Furthermore, the great variety of mutations and different prognostic markers related to AML also suggest that there are many different drivers or causes behind the heterogeneous phenotypes of AML that could be targeted. Thus, there is a great need for more personalized treatments that will target specific mutations and the defects associated with them (Estey, 2011).

Novel Mouse Model of AML:

To better understand leukemogenic mechanisms and analyze the effectiveness of preclinical treatments, the Caligiuri lab engineered a double knock-in mouse model of AML resulting from the co-presence of two human AML-associated mutations. The first mutation is

the *Mixed-lineage leukemia* gene "partial tandem duplication" (Mll^{PTD})[1] encoding an aberrant transcription factor with histone 3 lysine 4 (H3K4) methyltransferase activity (an epigenetic modification). Epigenetic regulation is the process by which gene expression is regulated without altering the actual nucleotide sequence of the gene. These epigenetic modifications are important in cancer when normal expression of genes is altered, either causing increased expression of oncogenes or reduced expression of tumor suppressors. The second mutation in our mouse model is the *fms-like tyrosine kinase* gene "internal tandem duplication" ($Flt3^{ITD}$), encoding a constitutively-active receptor tyrosine kinase. *FLT3* and *MLL* are key genetic loci in AML, and many labs are studying *MLL* fusion genes and $FLT3^{ITD}$ in the context of this disease. In this model, neither the single mutant $Mll^{PTD/WT}$ (referred to as Mll^{PTD}) mice nor the single mutant $Flt3^{ITD/WT}$ (referred to as $Flt3^{ITD}$) mice develop acute leukemia, and they live normal life spans. However, when the Mll^{PTD},$Flt3^{ITD}$ mutations are co-present in the mouse, i.e. "double mutant," 100% of these mice die within 50-60 weeks of age from AML (Figure 1; Zorko, 2012). The acute leukemia that develops can be transplanted into irradiated syngeneic recipient mice, resulting in highly aggressive acute leukemia and death within 60 days. The consistency of the transplant model makes it an ideal system for assessing drug efficacy in preclinical AML treatment trials. More importantly, our mouse model recapitulates important features of human AML creating an opportunity to study the underlying biology of AML.

Mixed Lineage Leukemia Gene:

The *MLL* gene is frequently mutated in AML and is considered an important prognostic marker for human patients. MLL is commonly fused to other proteins in leukemia in which the N-terminus of MLL remains, and the C-terminus is retained from its fusion partner (Eklund,

[1] Notation: MLL (human protein), *MLL (human gene).* Mll (mouse protein), *Mll (mouse gene).*

2011). Unlike fusion mutations in *MLL*, the *MLL*^{PTD} mutation is a mechanistic enigma in this field because the entire protein remains present rather than just a truncated form. The effect of the retained C-terminal SET domain in the partial tandem duplication is not fully understood. *MLL*^{PTD} is found in about 5% of cytogenetically normal AML patients, and it has been associated with a poor patient prognosis (Maward, 2012). Mutations of MLL are known to have epigenetic activity altering gene expression and leading to leukemogenesis. These epigenetic changes are a prime target for treatment in AML (Bernt, 2011). Unfortunately, H3K4 inhibitors that would directly target Mll are not yet available. Previous studies have shown that the wild type allele of MLL is epigenetically silenced in patients with MLL^{PTD}, and that reversing this silencing with DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) leads to leukemic cell death (Whitman, 2005).

FMS-Like Tyrosine Kinase:

FLT3 receptor tyrosine kinase (RTK) is normally associated with the regulation of cell proliferation, apoptosis, and maturation of myeloid progenitors and other hematopoietic cells. The *FLT3*^{ITD} mutated kinase is constitutively active, correlating with a high level of growth in AML blasts and poor prognosis for patients with this mutation (Beitinjaneha, 2010). Furthermore, activating mutations in *FLT3* are common in AML patients, accounting for approximately 30% of cases, and *FLT3* mutations are generally considered to be a driver of leukemic growth and proliferation in these patients (Pemmaraju, 2011). Other kinases have been shown to be good targets for inhibitors (eg. BCR-ABL in CML) and there are several small molecule inhibitors that target FLT3. Previously, receptor tyrosine kinase inhibitors (RTKi), such as PKC412, have been shown to prevent FLT3 activation and decrease the ability of

leukemia cells to survive (Weisberg, 2002). *FLT3*^{ITD} positive patients treated with RTKi's have shown response, but these patients often develop resistance and relapse (Illmer, 2007). Thus more potent and specific inhibitors of *FLT3*^{ITD} have since been developed, and one such RTKi, AC220, has been shown to selectively target and inhibit FLT3^{ITD} in human AML cell lines (Zarrinkar, 2009).

Treating AML with Epigenetic Modifiers and Tyrosine Kinase Inhibitors:

Since our mouse model mimics human AML, we used our *Mll*^{PTD},*Flt3*^{ITD} mice to study both the mechanisms behind AML survival and potential treatment targets. In a preliminary study on our mouse model, we found a global increase in DNA methylation in our leukemic *Mll*^{PTD},*Flt3*^{ITD} after sequencing methylated DNA captured with a methyl-binding protein (Figure 2). The use of hypomethylating agents and HDACi's in human patients has shown success in both myeloid dysplastic syndrome (MDS) and AML, but many patients still succumb to leukemia (McDevitt, 2012). Decitabine or 5AD, a DNA methyltransferase inhibitor, has shown activity in human AML patients (Blum, 2007), and AR42, novel histone deacetylase inhibitor (Lu, 2005), is currently undergoing clinical trial at The Ohio State University. We first hypothesized that a novel drug combination of epigenetic modifiers, 5AD and AR42, would improve survival in our *Mll*^{PTD},*Flt3*^{ITD} AML mouse transplant model. We demonstrate that treatment with epigenetic modifiers including 5AD and AR42 confers a survival benefit to *Mll*^{PTD},*Flt3*^{ITD} leukemic mice and temporarily corrects some of the molecular aberrations correlated with these mutations. However, all of the mice still die from leukemia. Since our mice also exhibit constitutive activation of the *Flt3*^{ITD} RTK, we hypothesized that combining epigenetic modifier treatment with inhibition of tyrosine kinases would further target leukemic

growth and increase survival in our mouse model. The RTKi's PKC412 and AC220 were shown to inhibit growth in both human AML cell lines and Mll^{PTD},$Flt3^{ITD}$ AML blasts cultured *ex vivo.* Despite promise *ex vivo,* AC220 treated mice exhibited toxicity and did not see an increase in survival *in vivo*. Further studies *ex vivo* on primary mouse AML blasts, suggest that a smaller, less toxic dose of AC220 may increase the efficacy of epigenetic modifiers. In the era of personalized medicine, these targeted treatment combinations may provide new treatment options for acute myeloid leukemia patients harboring Mll^{PTD},$Flt3^{ITD}$ mutations and improve their quality of life.

Chapter 2: Materials and Methods

Global Methylation:

A methyl-DNA binding domain was used to pull down methylated DNA fragments from non-leukemic samples and Mll^{PTD},$Flt3^{ITD}$ AML whole bone marrow. The pulled down fragments were sequenced and the Global Methylation Index for each sample group was calculated as described previously (Yan, 2012).

Growth Inhibition Assays:

In order to determine the ability of 5AD, AR-42, and AC220 to inhibit growth, human AML cell lines (MV4-11 and EOL-1) and primary Mll^{PTD},$Flt3^{ITD}$ mouse cells were treated *in vitro* with varying concentrations of both epigenetic modifiers and AC220/PKC412. The MV4-11 cell line contains an *MLL-AF9* fusion and $FLT3^{ITD}$ mutation, and EOL-1 cell lines contain MLL^{PTD} and *FIP1L1-PDGFRα.* Primary mouse cells treated *ex vivo* were cultured in IL-3 and rrSCF to maintain normal survival in culture.

Since 5AD is a nucleoside analog which must incorporate into DNA to inhibit DNA methyltransferases, cells were treated for 48 hours in order to see the full effect of 5AD. AR-42 and AC220 were started 24 hours after 5AD began. After treatment was complete, growth inhibition assays (CellTiter, Promega) were carried out to determine the level growth inhibition. MTS reagent was added to the culture (20ul/well) and incubated at 37°C for approximately one hour. A Thermo Multiskan Spectrum microplate reader was used to measure absorbance at 490 nm. After accounting for background spectrum, all growth was calculated as percentage of no treatment controls.

Apoptosis Assays:

In addition, apoptosis analysis was carried out on treated MV4-11 cells and primary mouse blasts through BD LSRII flow cytometer. Cells were stained with Annexin V (apoptosis marker) and 7-AAD (DNA stain) to measure the percentage of cells undergoing apoptosis associated with each treatment. All data was analyzed using FlowJo software.

***In Vivo* Mouse Treatment to Moribund State:**

Epigenetic Modifier Treatment: Wild type mice were sub-lethally irradiated and tail vein injected with one million primary *Mll*$^{PTD/wt}$ and *Flt3*$^{ITD/wt}$ mouse AML cells (obtained from spleen). 5AD, AR42, and vehicle control treatment began once the leukemia has fully engrafted (white blood cell count of >10,000 per µl of blood). 5AD doses were prepared fresh daily for treatments. 5AD was dissolved in a PBS solution, and mice were treated every day for four days through intraperitoneal (IP) injection at a dose of 0.2 mg/kg. AR42 was dissolved in 0.5% methylcellulose and 0.1% tween80, and mice were treated via oral gavage every other day over five days for a total of three doses at 50 mg/kg. The mice were monitored for general health and weight every day. When mice reached the moribund state, spleen, bone marrow, and other tissues were collected during sacrifice. There were at least six mice per treatment group. In addition to drug trials to moribund state, trials were performed in which mice were sacrificed 3/4 of the way through their drug treatment regimen. Samples collected from these mice were used to monitor pharmacodynamics endpoints such as spleen size, tumor suppressor expression, oncogene expression, and the presence of leukemic blasts in peripheral blood.

AC220 Tyrosine Kinase Treatment: Sublethally irradiated leukemic transplant mice were first treated with an AC220 dose of 10 mg/kg. Drug stocks were prepared in weekly batches and stored at 4°C. AC220 was dissolved in a 22% (2-Hydroxypropyl)-β-cyclodextrin solution. Mice

were treated through oral gavage every day for 28 days. Following the unsuccessful *in vivo* AC220 treatment trial, leukemic mice were given 30 mg/kg and 100 mg/kg of AC220 and sacrificed at 1 hour and 4 hours. Peripheral blood collected at the time of sacrifice was submitted to the Pharmacoanalytical Shared Resource to measure the effective plasma concentration at the time of sacrifice. After determining that a 30 mg/kg dose would provide an effective plasma concentration, a lethal irradiation of wild type mice was utilized for a more uniform engraftment across all mice. In addition to adding Mll^{PTD},$Flt3^{ITD}$ mouse AML cells, wild type BM cells were also added to rescue the mice from lethal doses of radiation. Treatment began when approximately 60% of peripheral blood was from the leukemic donor. Mice were treated through oral gavage every day for 30 days at a dose of 30 mg/kg for the second trial. As before all treated mice were monitored for health (including daily weight measurements), and half way through the trial each mouse received a one milliliter IP injection of phosphate buffered saline (PBS). When mice reached the moribund state, spleen, bone marrow, and other tissues were collected during sacrifice.

All *in vivo* drug studies were approved by IACUC.

Quantitative Real time RT-PCR:

Bone marrow samples were collected, and using an RNeasy Kit (Qiagen, Venlo, The Netherlands), mRNA was extracted from isolated Trizol (Invitrogen, Carlsbad, CA). mRNA was reverse transcribed to cDNA, and TaqMan probes for genes of interest were mixed with cDNA and run on an Applied Biosystems 7900HT Fast Real-Time PCR System to measure gene expression. All quantitative real time RT-PCR was quantified using the $^{\Delta\Delta}$Ct method, and linear mixed models were used for statistical analysis.

Immunoprecipitation and Western Bloting:

Primary mouse cells were lysed in RIPA solution with protease inhibitors after treatment *ex vivo* and *in vivo*. Antibodies for total Flt3 and IgG control (Cell Signaling Technology, Danvers, Massachusetts) were separately incubated in protein A/G beads followed by overnight rotation with protein lysates at 4°C. After overnight incubation, beads/lysates were washed with cold IP buffer, and the beads were resuspended in the desired SDS-PAGE Buffer. Samples were then run on precast 18 well gel (Biorad, Hercules, CA), and transferred onto a nitrocellulose membrane. Phosphorylation of RTK was probed using a pFLT3 or pSTAT5 antibody (Millipore, Billerica, Massachusetts). Films were exposed using ECL and Femto.

Statistical Analysis:

Synergistic action in all *in vitro* AML cell lines and primary mouse leukemia were performed according to previously described (Slinker, 1998). The Cox proportional hazard model was used in the *in vivo* drug studies to compare the survival between each treatment or vehicle controls, and Holm's method was used to control for multiple comparisons (3 comparisons).

Chapter 3: Results

Increased Global DNA Methylation Correlates with Increased DNA Methyltransferase Activity:

DNA methyltransferases or DMNTs are commonly dysregulated in leukemia and AML, and after conducting initial methylation studies on our Mll^{PTD},$Flt3^{ITD}$ leukemic mouse model, we asked whether the increase in global DNA methylation (1.7 fold verses non-leukemic control, p= 0.0174) in mice with leukemia correlated with a similar increase in *DNMT* mRNA expression. An increase in *DNMT* expression could explain aberrant DNA hypermethylation associated with Mll^{PTD}. Quantitative RT-PCR was performed on samples collected from age matched wild type, single mutant controls, and double knock-in leukemic mice (Figure 3). The mRNA expression of *Dnmt1, 3a*, and *3b* were all increased compared to the wild type controls (*Dnmt1*: 1.5 fold, p = 0.03; *Dnmt3a*: 2.3, p = 0.015; *Dnmt3b* 5.3 fold, p = 0.03). Along with the global hypermethylation observed in leukemic mice, these data supported our hypothesis that the addition of a hypomethylating agent (5AD) and an HDACi (AR-42) to increase gene expression of tumor suppressors may provide a therapeutic benefit *in vivo*.

Combination of Epigenetic Modifiers Targets Human AML Cell Lines *in vitro*:

In order to establish the efficacy of combination treatment of epigenetic modifiers in AML, we first tested the ability of 5AD and AR42 to inhibit the proliferation of AML *in vitro*. Two human AML cell lines were used: MV4-11, containing *MLL-AF4* and $FLT3^{ITD}$, and EOL-1, containing MLL^{PTD} and *FIP1L1-PDGFRα*. 5AD and AR42 were first tested as single agents to determine which concentrations produced significant growth inhibition through MTS assays. The twenty percent inhibitory concentration or IC_{20} was selected for each cell line (5AD: MV4-

11=3uM, EOL-1=1uM and AR42: Both=0.3uM). When the two epigenetic modifiers were combined at their IC_{20}, the drug combination was able to achieve a synergistic effect, inhibiting greater than 40% in each cell line. MV4-11 was the most sensitive to the epigenetic modifiers with a nearly 70% inhibition ($p<0.03$; Figure 4a). EOL-1 also achieved a similar synergistic effect with approximately 55% growth inhibition ($p<0.04$; Figure 4b).

In addition to testing the epigenetic modifier's ability to inhibit growth *in vitro*, we tested whether these doses were able to induce apoptosis in MV4-11 cells. Under the same conditions as the MTS assay, MV4-11 cells were stained with Annexin V and 7-AAD and visualized via flow cytometry. Annexin V stains phosphatidylserine which is exposed on the cell membrane only during apoptosis, and 7-AAD is a DNA stain that indicates cellular death. Treatment with both 5AD and AR42 achieved the greatest level of apoptosis (~15%) and death (~10%) when compared to either of the single drug or vehicle controls (Data Not Shown).

Since our mouse model mimics human AML well and provides us with a preclinical model to test potential treatments, we also tested the combination of 5AD and AR-42 on Mll^{PTD},$Flt3^{ITD}$ AML cells *ex vivo*. Mouse leukemic cells were cultured with two cytokines, rrSCF (recombinant rat Stem Cell Factor) and IL-3 (Interleukin 3), to promote normal survival and growth *ex vivo*. 5AD and AR42 that caused minimal levels of growth inhibition as single agents (5AD: ~30%, $p<0.0001$ vs. vehicle; AR42: ~40%, $p<0.0001$ vs. vehicle). Similarly to results seen in human AML cell lines, the primary mouse leukemic blasts were most sensitive to a combination of 5AD and AR-42 with a growth inhibition of greater than 60% ($p<0.0001$ vs. vehicle or vs. 5AD and $p=0.005$ vs. AR42, Figure 4c).

Epigenetic Modifiers Increase Survival of *Mll*^{PTD},*Flt3*^{ITD} Mice *in vivo*:

After demonstrating the efficacy of combining epigenetic modifies *in vitro,* we moved to treatment in the *Mll*^{PTD}, *Flt3*^{ITD} mouse model. We transplanted primary whole spleen cells collected from a primary *Mll*^{PTD},*Flt3*^{ITD} leukemic mouse into a subleathally irradiated syngeneic wild type mice. In order to ensure full engraftment of AML, leukemia was confirmed in transplant mice when the total average white blood cell count (WBC) was greater than 10,000/ul for each treatment group. After engraftment of the AML was confirmed, one round of treatment was performed. 5AD was dosed at 0.2 mg/kg every day for four days, and AR42 was dosed at 50 mg/kg every other day for a total of three doses. Placebo or vehicle treated mice reached the moribund state at a median of 36 days since transplantation of AML. 5AD treatment alone did not show a significant increase in survival with a median survival of 41 days (p = 0.1058), but treatment with AR42 alone showed a significant increase in survival with a median of 46 days (p = 0.0002 vs. vehicle). In order to determine if the order of treatment altered survival in our mice, we tested sequential treatment of 5AD followed by AR42 compared to AR42 followed by 5AD. No difference was observed when treated with 5AD or AR42 first. The combination of 5AD and AR42 displayed the most significant increase with a median survival of 51.5 days ($p < 0.0001$ vs. vehicle). The combination of epigenetic modifiers not only displayed a significant increase compared to vehicle, but it also increased survival compared to either of the single drug controls (p=0.0039 vs. AR42 (46d0; p<0.0001 vs. 5AD (41d)). While treatment with both epigenetic modifiers was able to increase survival, all mice eventually succumbed to leukemia (Figure 5).

Epigenetic Modifiers Reduce AML Blast Load *in vivo*:

AML in our *Mll*^{PTD}, *Flt3*^{ITD} mouse model typically exhibit an increase of leukemic blasts in the peripheral blood and splenomegaly as blasts infiltrate the spleen. In order to assess the effectiveness of our treatment with epigenetic modifiers, we sacrificed a group of AML transplant mice 3/4 of the way through their treatment with 5AD, AR-42, or both. This enabled us to observe any changes that occur during treatment. Peripheral blood smears were stained with a Wright-Giemsa stain to visualize the level of leukemic blasts present in the blood. Mice treated both with single drugs and the combination of epigenetic modifiers all displayed a marked reduction in AML blasts in their peripheral blood when compared to an untreated control (Figure 6a). Spleen weights increase overtime as AML progresses, and leukemic blasts infiltrate the spleen. Similarly to the effects seen in peripheral blood smears, mice treated with single drugs or both epigenetic modifiers saw an approximately two fold reduction in spleen weight ($p = 0.003$; Figure 6b). While the combination of epigenetic modifiers had the greatest increase in survival, single drug and combination treatment equally reduced leukemic blast burden in our mouse model, suggesting that the combination further increased survival through another mechanism. Ultimately, treatment with epigenetic modifiers was able to effectively target AML blasts in our mouse model.

Epigenetic Modifiers Increase Expression of Tumor Suppressor in Mouse Model:

In order to better understand the ability of treatment with epigenetic modifiers to reverse epigenetic silencing of tumor suppressors in our mouse model, we cultured primary AML blasts *ex vivo*, treating with varying doses of 5AD and AR42 both alone and in combination. AR42 alone was able to increase the expression of two cell cycle arrest and differentiation regulators:

Rb and *Ndrg1* (Figure 7a/b). Furthermore, the promoter of cell cycle regulator *Cdkn1a* is hypermethylated in bone marrow of leukemic mice when compared to non-leukemic mice (Data Not Shown). After treatment with the epigenetic modifier combination, *Cdkn1a* expression increased three fold when compared to vehicle treated controls (p=0.026, Figure 7c). This data suggest that treatment with 5AD and AR42 is able to reverse aberrant epigenetic alterations in leukemic cells and lead to the re-expression of tumor suppressors. This may account for the increased survival observed in the combination treated mice, as cell growth is inhibited through *Cdkn1a*.

Epigenetic Modifiers Reduce Oncogene Expression in Mouse Model:

After seeing an increase in tumor suppressor expression after treatment, we asked whether the aberrant regulation of MLL targets was also targeted with treatment. *HoxA9* is a direct target of Mll and highly upregulated in Mll^{PTD} mice (Zorko, 2012). RNA was obtained from bone marrow samples collected at the time of sacrifice during *in vivo* studies, and *HoxA9* expression was reduced by three fold after treatment with both epigenetic modifiers (p = 0.0038, Figure 8a). *DNMT3a* and *DNMT3b* are DNA methyltransferases associated with de novo DNA methylation. Like *HoxA9*, both are abnormally upregulated in Mll^{PTD},$Flt3^{ITD}$ leukemic mice (Zorko, 2012). After treatment with 5AD and AR-42, *Dnmt3a* and *Dnmt3b* exhibited 60% and 75% expression reduction respectively ($p < 0.05$ and $p < 0.003$; Figure 8b). Additionally, AR42 treatment alone achieved nearly 50% reduction in *DNMT3b* expression ($p < 0.003$; Figure 8b). Treatment with 5AD and AR42 reduced expression of *DNMT1* (associated with DNA methylation maintenance) by over 45% ($p < 0.05$; Figure 8b). These data suggest that treatment

with epigenetic modifiers targets the upregulation of oncogenes associated with Mll^{PTD} possibly contributing to the antileukemic effects of this treatment combination.

Epigenetic Modifiers Induces Differentiation in Mll^{PTD},$Flt3^{ITD}$ Leukemic Cells:

In addition to correcting aberrant expression of tumor suppressors and oncogenes, we asked whether treatment with epigenetic modifiers would induce differentiation of leukemic blasts. AML is often characterized by an increase in immature, abnormal white blood cells. Due to a block in differentiation, these blasts or progenitor cells accumulate in the peripheral blood crowding out the development of healthy blood cells (Gocek, 2011). Induction of differentiation may inhibit some of the growth potential of AML blasts and may represent less toxic therapy than leukemic death due to cytotoxic drug effects. Studying cells collected from mice 3/4 of the way through treatment, we saw an increase in cell surface expression of CD11b (data not shown) and GR1 (Figure 9) both of which are markers linked to myeloid cell differentiation.

Tyrosine Kinase Inhibitors Effectively Target $FLT3^{ITD}$ Positive Human AML Cell Lines:

While treatment with epigenetic modifiers increased mouse survival and reduced many of the molecular effects associated with AML in our model, all of the mice still succumbed to leukemia. Since much of our treatment with epigenetic modifiers was targeted to downstream effects associated with the MLL^{PTD} and $FLT3^{ITD}$ mutations, our treatment regimen had not directly targeted either mutation. Therefore, we examined the ability to effectively target mutated FLT3 RTK using two different tyrosine kinase inhibitors: PKC412 and AC220. Both of these inhibitors have been shown to have activity against $FLT3^{ITD}$, and both have been used to treat AML in human patients. We compared the efficacy of these two inhibitors on the $FLT3^{ITD}$

positive AML cell line MV4-11. Cell proliferation assays showed activity at inhibiting leukemic growth *in vitro* with an IC_{50} of 46.8 nM for PKC412 and 3.2 for AC220 (SE: 7.962 and 0.72 | 95% CI: 31.0-62.7 and 1.68-4.72; respectively; Figure 10a). Furthermore, these drugs induced apoptosis in MV4-11 when compared to vehicle with PKC412 requiring approximately 10 fold higher dose to achieve the same level of cell death as AC220 (Data Not Shown). Finally, we tested the ability of the two inhibitors to reduce phosphorylation of FLT3 (pFLT3). Receptor tyrosine kinase molecules are phosphorylated during activation, and thus, a reduction in pFLT3 would indicate a deactivation of its signaling pathway. MV4-11 cells were lysed after treatment with the two RTKi's, and an immunoprecipitation (IP) of total FLT3 was performed to observe changes in pFLT3. PKC412 was able to achieve a high level of pFLT3 reduction with a dose of 300 nM, but AC220 saw no pFLT3 at doses as low as 1 nM (Figure 10b). These data lead us to conclude that AC220 would be a far better drug choice to target $Flt3^{ITD}$ in our mouse model.

In order to determine if our primary Mll^{PTD},$Flt3^{ITD}$ cells were sensitive to inhibition of Flt3, we cultured blasts *ex vivo* and treated with varying doses of PKC412 and AC220. Unlike MV4-11 cells which had low IC_{50} values, primary mouse AML cells were not as sensitive. Doses as high as 1,000 nM were required of both AC220 and PKC412 to achieve a 50% growth inhibition (Figure 10c).

Low Dose of AC220 Unable to Increase Survival of Mll^{PTD},$Flt3^{ITD}$ Mice *in vivo*:

Zarrinker et al showed that treatment of an MV4-11 xenograft with 10mg/kg AC220 significantly increased survival (Zarrinkar, 2009). Therefore, we used this same dose to measure efficacy on our transplant mouse model of Mll^{PTD},$Flt3^{ITD}$ leukemia. In this trial, there was no significant increase in survival compared to the vehicle treated mice (Figure 11a).

We next asked why this dose was ineffective *in vivo*, but we were unable to successfully detect pFlt3 after IP from flash frozen bone marrow or spleen cells after treatment with AC220. However, Stat5, a known downstream target of Flt3 (Choudhary, 2005), was still phosphorylated after treatment. This is in contrast to a reduction in pSTAT5 seen when MV4-11 cells are treated *in vitro* (Figure 11b). The failure to inhibit the phosphorylation of Stat5 suggests that AC220 did not adequately target Flt3 activation in treated mice.

Earlier studies *ex vivo* demonstrated that our mouse model was not nearly as sensitive to RTKi's as MV4-11 cell line, potentially explaining why a dose of 10 mg/kg may have been effective in a MV4-11 xenograft but not our mouse model. In order to determine what dose would deliver enough AC220 to reach a plasma concentration within the range of RTKi sensitivity of our $Mll^{PTD},Flt3^{ITD}$ mouse AML, we performed a small pharmacokinetic (PK) study comparing the plasma concentration at 1 and 4 hours after a dose of 30 or 100 mg/kg AC220 (Figure 11c). The results of this PK trial demonstrated that a dose of 30 mg/kg would achieve a plasma concentration of 7.45 and 13.63 uM (1 and 4 hours respectively). These concentrations were well within the effective range of AML cell inhibition (1,000 – 10,000 nM) demonstrated with AC220 *ex vivo*. These data indicate that using a dose of 30 mg/kg may more successfully target and inhibit $Flt3^{ITD}$ in our mouse model.

Higher Dose of AC220 in $Mll^{PTD},Flt3^{ITD}$ Mice *in vivo* Leads to Drug Toxicity:

Since higher doses of AC220 showed promise on our mouse AML cells *ex vivo*, we started a second *in vivo* trial with a dose of 30 mg/kg. To ensure a greater uniformity in blast engraftment by delivering the same ratio of AML to WT cells, we performed a lethal irradiation

transplant, and in order to prevent death from radiation, we transplanted wild type bone marrow in addition to the *Mll*^{PTD},*Flt3*^{ITD} leukemia.

After an initial gain in weight gain compared to vehicle controls, all treated mice began to quickly lose weight starting ten days after treatment initiation. On day ten, peripheral blood bleeds showed anemia in 75% of AC220 treated mice, with no sign of leukemia in treated or control mice. One treated mouse died on day 17 with high levels of leukemia after flow cytometry analysis showed a WBC of >100,000/ul. The drug trial was suspended two days later after two more treated mice lost greater than 10% of weight meeting early removal criteria (Figure 12a). Spleen and bone marrow samples were collected at time of sacrifice and stained for Ly5.1/Ly5.2. Since our *Mll*^{PTD},*Flt3*^{ITD} mouse model has a Ly5.2 background, we transplant into Ly5.1 mice in order to easily distinguish leukemic blasts from wild type cells via flow cytometry. In AC220 treated mice, both bone marrow (data not shown) and spleen showed a 1.6-fold in percent Ly5.1 cells when compared to vehicle controls (p=0.0089, Figure 12b); furthermore, a corresponding decrease in Ly5.2 leukemic cells (1.6-fold, p=0.0089) was also observed. These results suggest that while AC220 was toxic at the dose used, it still has some antileukemic affect.

Tyrosine Kinase Inhibitors Enhance the Antileukemic Effect of Epigenetic Modifiers *in vitro*:

While AC220 showed toxicity in our mouse model at a higher dose, we did see a promising increase in percentage of wild type cells in the spleen compared to vehicle controls suggesting this drug was targeting AML blasts. After showing success as a single agent on both human AML cell lines and primary *Mll*^{PTD},*Flt3*^{ITD} cells *in vitro*, we asked whether the addition

of our epigenetic modifier regimen would increase the efficacy of a tyrosine kinase inhibitor and allow a lower dose in our mice. In order to test this hypothesis, we examined the ability of epigenetic modifiers and AC220 to inhibit growth in MV4-11 cell lines. Using moderately effective doses of 5AD and AR42 (1,000 nM and 200 nM respectively), MV4-11 cells were treated *in vitro* with varying combinations of epigenetic modifiers and the tyrosine kinase inhibitor AC220 (10-0.3 nM). Because of the toxicity seen *in vivo*, we tested AC220 at levels that on its own would cause between 0-15% growth inhibition. When combining these AC220 doses with 5AD and AR42 (~60%, p=0.0091), the resulting triple drug combination exhibited 78% inhibition (Figure 13a, p=0.0049). In addition, the triple concentration of 5AD, AR42, and AC220 produced a modest increase in apoptosis when compared to treatment with vehicle (58%, p=0.0008), and the triple combination produced a significant increase in cell death when compared with epigenetic modifiers only (20.0% vs. 12.1%, p=0.0471; Figure 13b). To better determine the efficacy of this triple drug combination in our mouse model, we performed preliminary studies on primary Mll^{PTD},$Flt3^{ITD}$ blasts *ex vivo*. Similarly to results seen in MV4-11, the addition of AC220 (1,000 nM) to epigenetic modifiers (5AD 1,000 nM and AR42 100 nM) was able to induce a greater level of growth inhibition (90%, Figure 13c) and apoptosis (32.9%, Figure 13d) than treatment with the vehicle. These results suggest that a smaller dose of AC220 may increase the efficacy of 5AD and AR42 when combined in our mouse model, possibly circumventing the toxicity observed at higher doses.

Chapter 4: Discussion

Personalized medicine and targeted cancer therapies are the future of medicine. This project aimed to elucidate the ability of a novel combination of epigenetic modifiers and a tyrosine kinase inhibitor to bring *MLL*^{PTD} and *FLT3*^{ITD} patients closer to obtaining specific, targeted therapy. Combining 5AD and AR42 not only synergistically inhibited growth *in vitro* on two human AML cell lines but also increased apoptosis in these same cells. Treatment with the epigenetic drug combination *in vivo* was able to significantly increase survival, and mice sacrificed in the midst of treatment with epigenetic modifiers displayed a marked reduction in overall leukemic burden. In addition to conferring a survival benefit, treatment with epigenetic modifiers were able to increase expression of the cell cycle regulator *Cdkn1a* which is hypermethylated in our leukemic mice. Together these results indicate that this combination treatment is able to target some of the downstream effects of *MLL*^{PTD} and *FLT3*^{ITD} thus increasing survival.

With the current standard of care, human AML patients frequently relapse after therapy (Burnett, 2011). Similarly, in my experiments with epigenetic modifiers, all of the treated mice succumbed to AML, presumably because the epigenetic modifiers did not fully target and kill the leukemia stem cells (or leukemia initiating cells). In humans, these leukemia stem cells (LSC) are more resistant to treatments and are generally considered to cause relapse in patients (Bruss, 2011). Increased expression of the surface cell markers CD11b and GR1 are signs that treatment with epigenetic modifiers leads to an induction of differentiation. These results could indicate that some populations of leukemia stem cells are differentiated, but due to the small number of leukemia stem cells and blood progenitor cells collected at the time of sacrifice, it is unclear whether this treatment is able to specifically target leukemia stem cells. One obstacle to

targeting LSCs in human patients has been their ability to evade treatment and acquire resistance. There are many different potential mechanisms by which LSCs survive chemotherapy and other treatments: LSC quiescence, plasma membrane drug pumps, and protection in the "stem cell niche" of the bone marrow (Valent, 2011). Slower growth or quiescence of these leukemic stem cells may allow them to evade the effects of drugs targeting actively growing cells. As a nucleoside analog, 5AD requires active DNA replication and division of cells to achieve inhibition of DNA methyltransferase. If the LSCs are not dividing during the treatment period, they will not be targeted by 5AD and will repopulate the blood with AML blasts. Similarly, an upregulation of drug pumps in LSCs could lead to an efflux of drugs out of the AML cells leading the epigenetic modifiers to have a minimized effect. The "stem cell niche" is the idea that LSCs congregate to a protected microenvironment in the bone marrow where drugs are unable to reach these cells. These possible LSC functions could explain the method by which our epigenetic modifiers increased survival in our mouse model but did not achieve a complete remission. An important future area of study should look into our ability to target these stem cells and increase their sensitivity to these drugs to enhance survival after treatment with epigenetic modifiers. Furthermore, the observed induction of differentiation could heighten leukemic blasts' sensitivity to other chemotherapeutic agents as seen in acute promyelocitic leukemia (APL) when treated with ATRA (Cassinat, 2001).

Another important area of future research could be the role of altered epigenetic modifications in both our mouse model and human AML. These studies could address the question of whether altered epigenetics are the cause of leukemia or are simply an effect of leukemic initiation. If epigenetic modifications are only necessary for the leukemogenesis process, rather than maintenance, their reversal may not be sufficient to kill leukemic blasts. In

this case, the hypermethylation observed is not necessary for the survival and proliferation of AML, and thus treatment seeking to reverse these changes may not provide a significant survival increase. Since we demonstrated the increased expression of multiple tumor suppressors after treating primary mouse cells, this scenario is less likely, but another possibility is that if these epigenetic modifications are just byproduct of AML in general. Leukemia in our model may be driven by other targets other than epigenetic modifiers such as the constitutive activation of the Flt3 pathways or other deregulated tyrosine kinase pathways. In this case, the epigenetic modifier treatment may simply be targeting an effect of cancer rather than its cause. Ultimately, additional treatment targets may be necessary to effectively treat leukemia in patients with these abnormalities.

The activating $FLT3^{ITD}$ mutation found in our model and many human patients is another potential target to develop new treatments for AML. Receptor tyrosine kinase inhibitors like PKC412 and AC220 could further increase survival targeting the direct effects of FLT3 constitutive activation. As previously demonstrated, both RTKi's were able to inhibit AML cell growth and reverse phosphorylation of FLT3 in human AML cell lines positive for $FLT3^{ITD}$. AC220 proved to be a far more potent drug to target the mutant tyrosine kinase, and because of these promising results *in vitro,* we moved forward in testing AC220 in our Mll^{PTD},$Flt3^{ITD}$ transplant AML mouse model. After performing a small PK study of AC220 in mice, we determined that a dose of 30 mg/kg would be sufficient to generate plasma concentration levels needed to kill AML *in vivo.* Unfortunately, this high dose of AC220 caused general toxicity with the treated mice succumbing to anemia well before any of the vehicle treated mice exhibited significant AML symptoms, but 75% of the treated mice did see a reduction of leukemic blast

infiltration in their spleen and bone marrow. These results indicate that at the doses used, AC220 was too toxic to increase survival in our mouse model.

There are a few possibilities that could explain the unsuccessful trial in our model. First, it is possible that differences between human $FLT3^{ITD}$ and mouse $Flt3^{ITD}$ render AC220 less effective against the mouse tyrosine kinase. The original papers characterizing AC220 were conducted in an MV4-11 xenograft mouse model of AML. It is possible that this cell line is particularly sensitive to AC220 because its growth is driven by $FLT3^{ITD}$ more so than our mouse model. Since current clinical trials with AC220 as a single agent have shown efficacy in human AML patients (Levis, 2012), it is more likely that mouse $Flt3^{ITD}$ is less sensitive to the drug. While AC220 is specific to FLT3 and KIT (another RTK important to AML and our mouse model), it is possible that the doses used *in vivo* during our mouse trial inhibited off targets important to growth of normal blood cells (KIT could be important due to its presence in hematopoietic stem cells). Another possibility for AC220's failure in our studies is that the specific $Flt3^{ITD}$ mutation in our mouse model is resistant to AC220. A recent paper demonstrated that human AML patients, who initially responded well to AC220 but later developed resistance, had three specific mutations in FLT3 which prevented the tyrosine kinase inhibitor from working effectively (Pauwels, 2012 and Albers, 2013). While our model does not express these specific mutations, it is possible that our mice have another $Flt3^{ITD}$ mutation that is resistant to AC220 or have acquired these specific mutations over time. Further studies could be carried out to determine whether a smaller dose could achieve better survival *in vivo,* but until the exact reason for the drug's failure in our model is fully known, these alterations could prove futile.

While AC220 did not prove successful as a single agent in our mouse model, we were able to show that a combination of epigenetic modifiers and tyrosine kinase inhibitors did provide a significant increase in the ability to inhibit leukemic cell growth *in vitro*. In both MV4-11 cells and primary *Mll*^{PTD},*Flt3*^{ITD} mouse cells, 5AD and AR42's killing potential was enhanced by the addition of AC220 or PKC412. Since these drugs worked well together *in vitro*, it is possible that the addition of AC220 at a smaller dose that does not induce toxicity could further enhance the antileukemic effect observed in our mouse model when treating with 5AD and AR42. Even if AC220 is ultimately unsuccessful in our mouse model, it may still be effective in human patients as an added component to treatment with epigenetic modifiers. Since AC220 is currently showing promise in clinical trials, its ability to target AML could be enhanced with the addition of 5AD and AR42 if patients exhibit hypermethylation or other epigenetic modifications.

The ultimate goal of this research was to develop personalized therapies for patients with AML. The use of AC220 to directly target the constitutive activation of FLT3^{ITD} RTK certainly qualifies as a potential personalized treatment, but the epigenetic modifier combination of 5AD and AR42 does not specifically target either of the mutated proteins in our model. While both MLL^{PTD} and FLT3^{ITD} are associated with epigenetic alterations in AML, these two drugs do not specifically target the mutations themselves, but rather, 5AD and AR42 globally target DNA hypermethylation and the deacetylation of histones. In order to achieve a true personalized medicine model for MLL^{PTD} and FLT3^{ITD} AML patients, direct inhibitors of MLL^{PTD} will be necessary. As previously stated, no direct inhibitors of MLL's methyltransferase activity have been developed. In fact, the presence of multiple H3K4 methyltransferases may make that design difficult. However, we have considered targeting other members of the MLL complex.

We have shown that *Meis1*, a direct target of *MLL*, is upregulated in our *Mll*^{PTD},*Flt3*^{ITD} leukemic mice. As expected Mll exhibits greater occupancy at the *Meis1* promoter; however, there is not a corresponding increase in H3K4 methylation despite the presence of a SET domain. Instead, the *Meis1* promoter shows increased H3K79 methylation, suggesting that Mll^{PTD} may recruit an H3K79 methyltransferase to activate the *Meis1* promoter. Previous studies have shown that the H3K79 methyltransferase DOT1L is required for activity of MLL fusion proteins in leukemogenesis (Ngyuyen, 2011). In addition, potent inhibitors of DOT1L have been tested in MV4-11 xenograft mouse models and shown to significantly increase survival (Daigle, 2011). These previous studies indicate that targeting of aberrant MLL^{PTD} function through inhibition of DOT1L may provide a therapeutic benefit in our mouse model. This treatment strategy would represent a truer personalized approach than epigenetic modifier combinations described in this paper. If successful as a single agent a DOT1L inhibitor could be combined with AC220 to dually target both MLL^{PTD} and FLT3^{ITD}.

AML is a highly heterogeneous and diverse form of cancer, and it is unlikely that one single drug will provide a therapeutic benefit to every patient. Thus there is a great need to develop drugs and treatment regimens which target the specific mutations or the downstream effects associated with a particular patient. This more personalized approach to treating AML could not only improve the quality of life but also increase survival rates in human patients. In this study, we demonstrate the potential of combining two epigenetic modifiers, 5AD and AR42, in a novel mouse model of AML containing the *Mll*^{PTD},*Flt3*^{ITD} mutations. Furthermore, preliminary studies indicate that the addition of a receptor tyrosine kinase inhibitor could enhance the ability of 5AD and AR42 to target and kill AML blasts. Ultimately, this

combinatorial effect may provide a novel personalized therapy for *MLL*^{PTD} and *FLT3*^{ITD} positive patients who do not respond well or are not candidates for current treatment methods.

Figures:

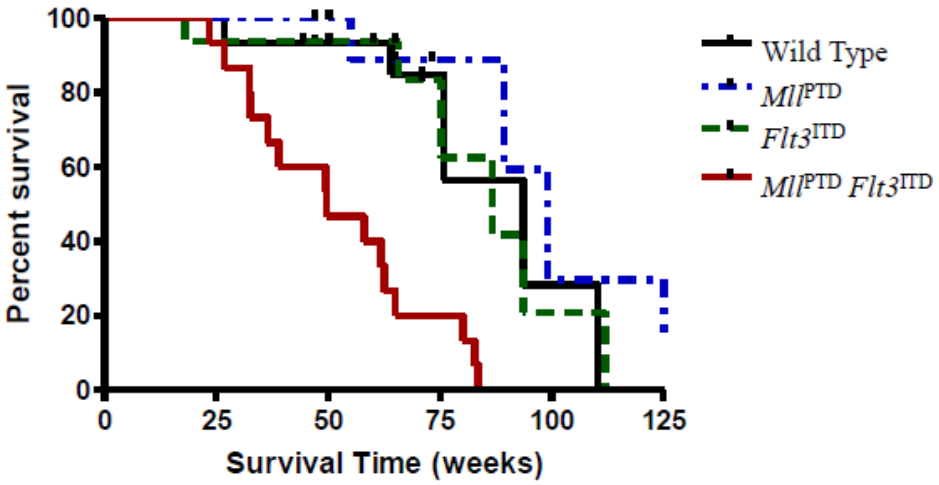


Figure 1 *Mll^{PTD},Flt3^{ITD}* Mouse Model Survival: Kaplan-Meier survival curve demonstrates that both the single mutant *Mll*^{PTD} or *Flt3*^{ITD} mice do not have any significant change in survival when compared to wild type controls. Conversely, 100% of the double mutant mice with one copy of both *Mll*^{PTD} and *Flt3*^{ITD} had significantly reduced life spans with a median survival of 49 weeks verses 75-94 weeks for the controls ($p<0.0003$). (Zorko, 2012)

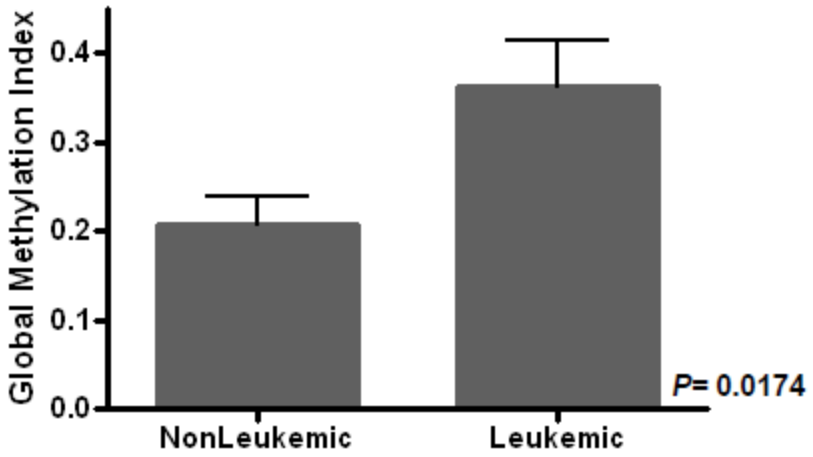


Figure 2 *Mll*^{PTD}, *Flt3*^{ITD} Mice Have an Increase in Global DNA Methylation Index: Global DNA hypermethylation index was observed in *Mll*^{PTD}, *Flt3*^{ITD} leukemic whole bone marrow samples (n=8) and compared to non-leukemic controls (n=7). The DNA methylation index was calculated based on comparison of pulled down and sequenced methylated DNA fragments in comparison to CpG frequency in the genome.

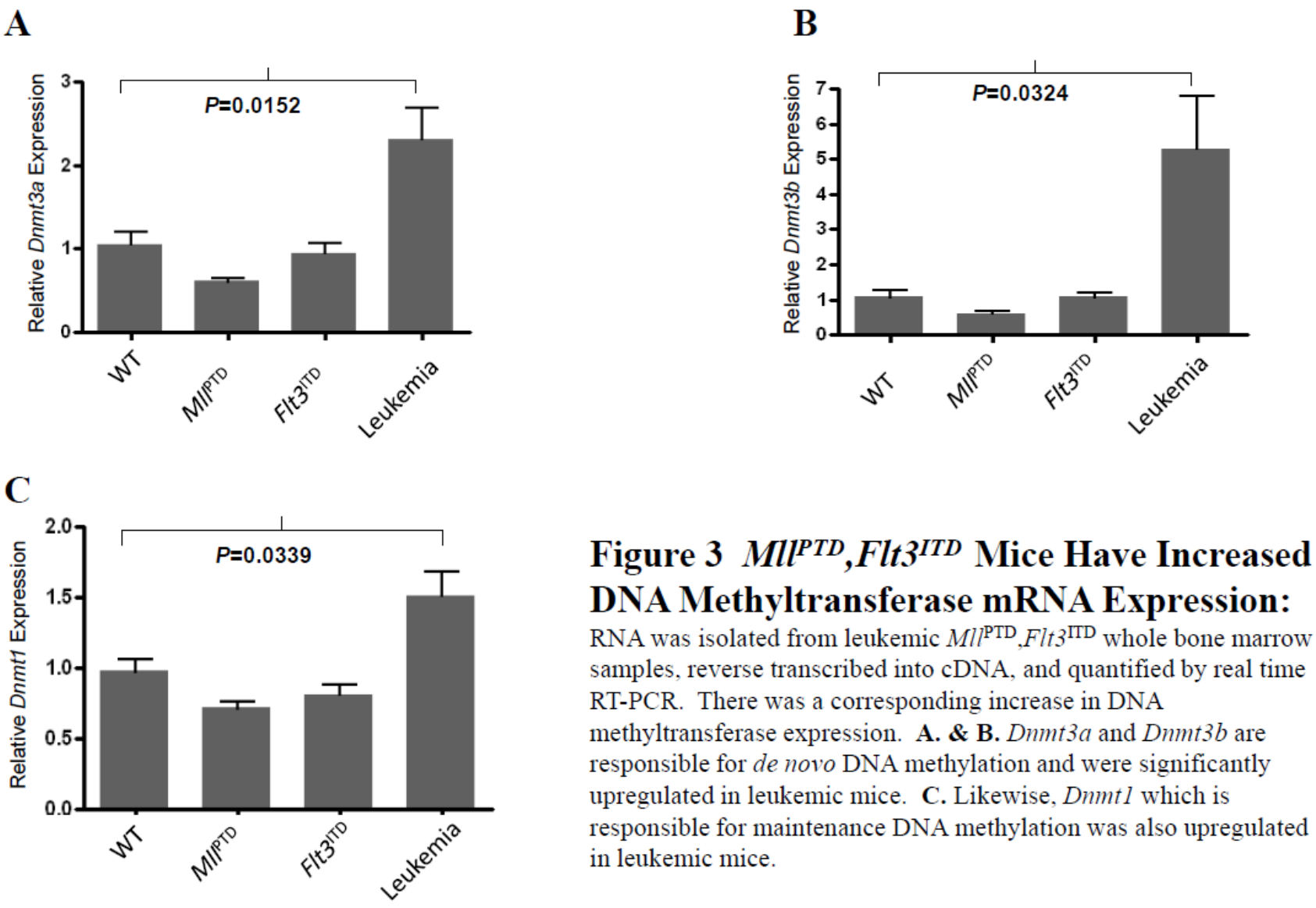
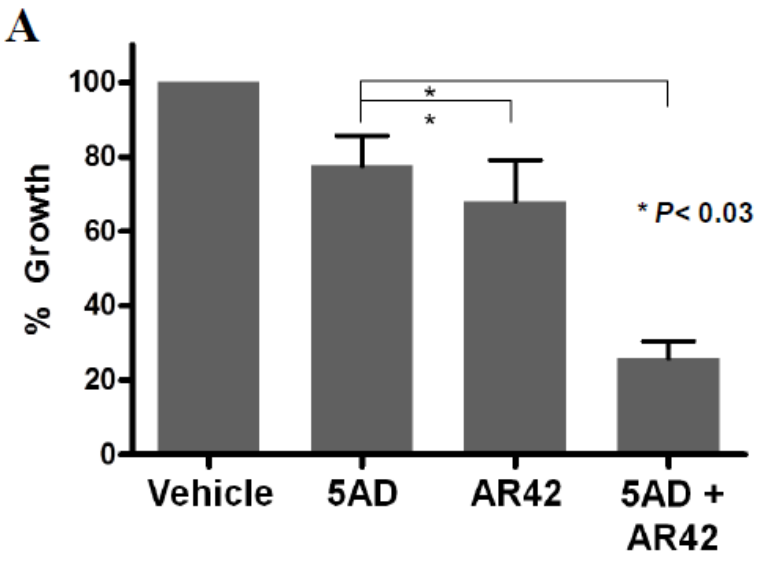
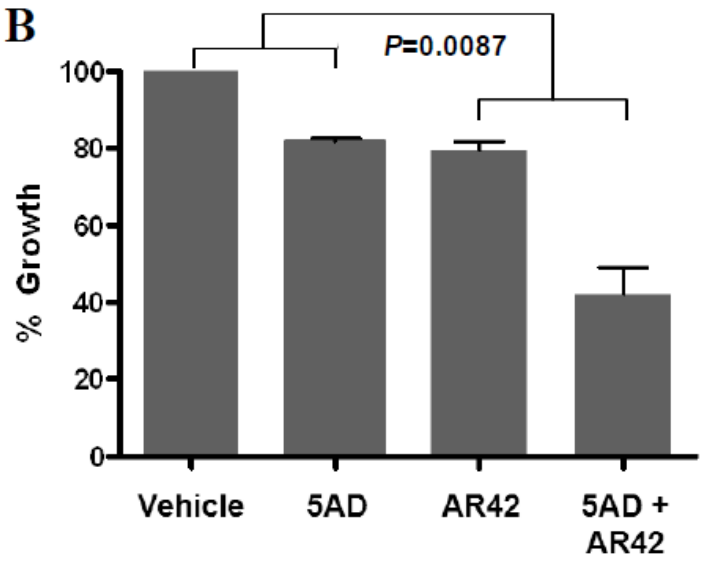


Figure 3 *Mll*^{PTD},*Flt3*^{ITD} Mice Have Increased DNA Methyltransferase mRNA Expression:

RNA was isolated from leukemic *Mll*^{PTD},*Flt3*^{ITD} whole bone marrow samples, reverse transcribed into cDNA, and quantified by real time RT-PCR. There was a corresponding increase in DNA methyltransferase expression. **A. & B.** *Dnmt3a* and *Dnmt3b* are responsible for *de novo* DNA methylation and were significantly upregulated in leukemic mice. **C.** Likewise, *Dnmt1* which is responsible for maintenance DNA methylation was also upregulated in leukemic mice.





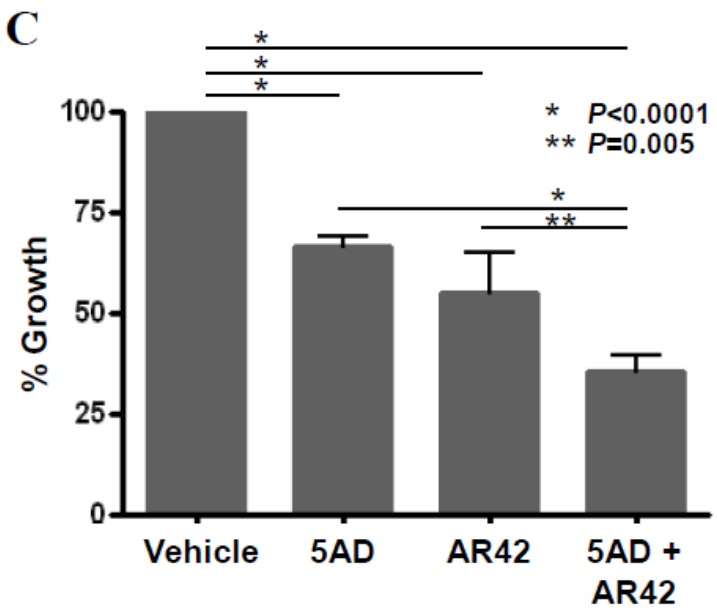


Figure 4 Epigenetic Modifiers Inhibit Leukemic Growth *in vitro*: Prior to treating *in vivo*, 5AD and AR42 growth inhibition studies were carried out on two human AML cell lines *in vitro* and primary Mll^{PTD},$Flt3^{ITD}$ leukemic blasts *ex vivo*. Cells were either treated with 5AD or vehicle, followed by AR42 or vehicle 24 hours later (cells were treated for a total of 48 hours). This yielded four different conditions: Vehicle, 5AD alone, AR42 alone, and 5AD & AR42. Growth was measured using MTS growth inhibition assays **A.** MV4-11 cells treated with the combination saw a synergistic growth inhibition of nearly 70% with a combination of AR42 and 5AD. **B.** Likewise, EOL-1 cells saw an approximately 55% synergistic growth inhibition when treated with the combination. **C.** Primary mouse leukemia was cultured *ex vivo* to ensure that these drugs were effective in our mouse model. Like the results seen in human cell lines, the combination showed the greatest level of growth inhibition.

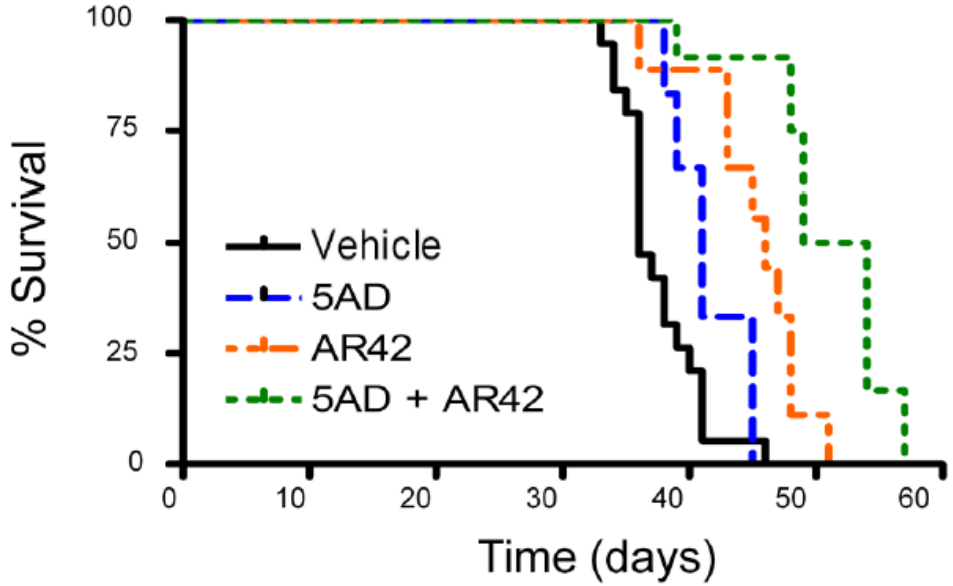


Figure 5 Survival Increases in *Mll^{PTD},Flt3^{ITD}* Transplant Mice with Epigenetic Modifier Treatment: Spleen cells from leukemic mice were transplanted into sublethally irradiated syngeneic mice. This Kaplan-Meier survival curve shows that vehicle control mice survived to a median of 36d post transplant (n=19). 5AD did not significantly increase survival with a median of 41d (n=6, p=0.1058). AR42 alone increased survival to a median of 46d (n=9, p=0.0002). Together, 5AD + AR42 yielded the greatest survival increase with a median survival of 51.5d (n=6, p<0.0001). Since there was no difference observed between mice treated with 5AD followed by AR42 compared to AR42 followed by 5AD, these mice were combined into one group labeled 5AD+AR42. All mice eventually succumbed to leukemia. Dosing: 5AD (0.2mg/kg qd4) AR42 (50mg/kg qod5)

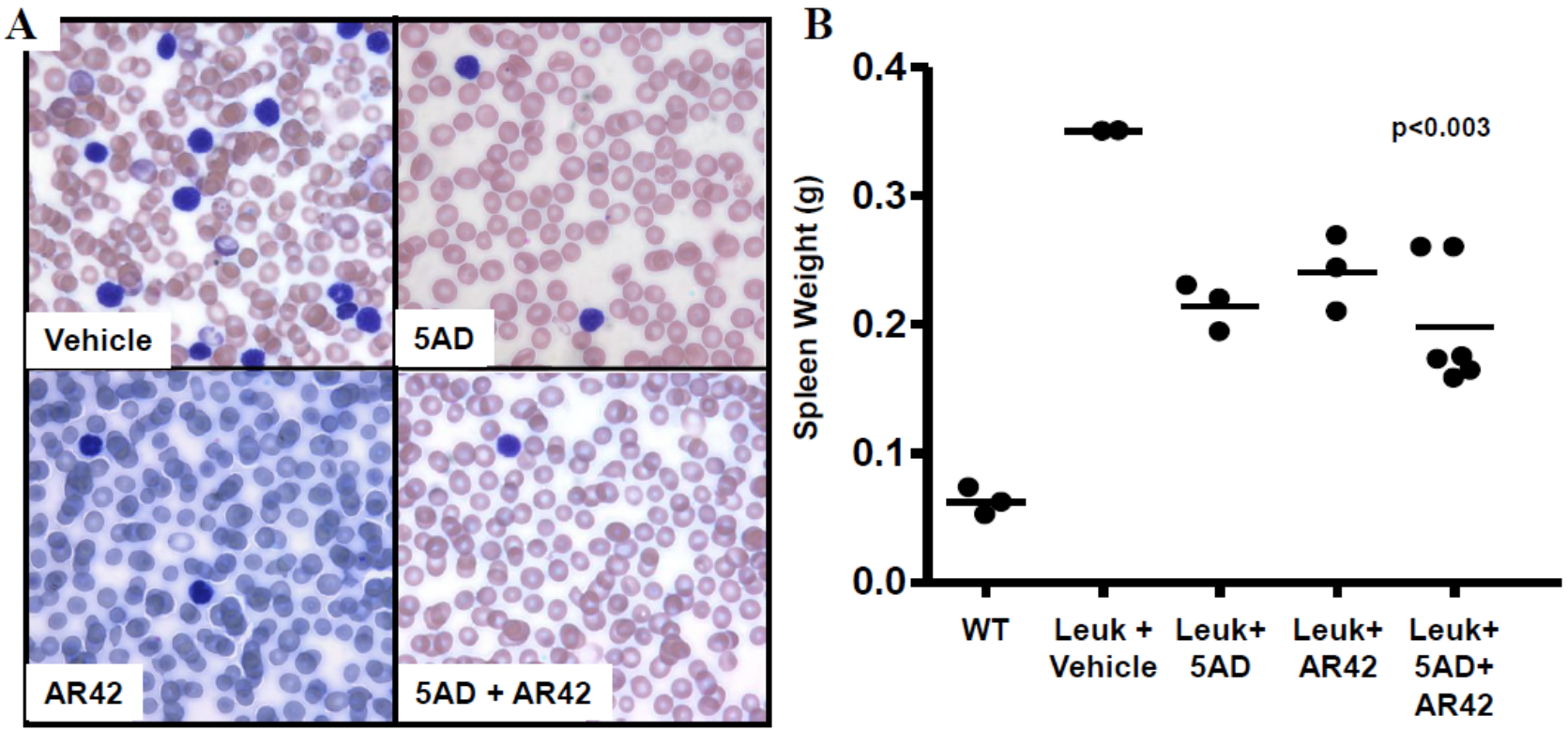
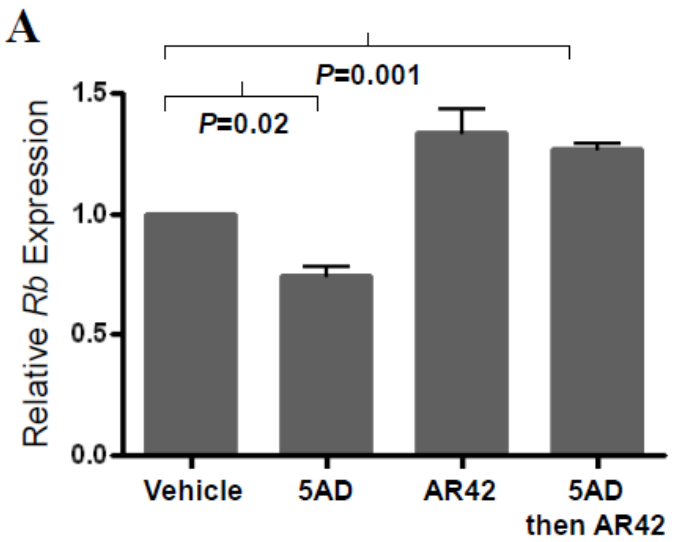
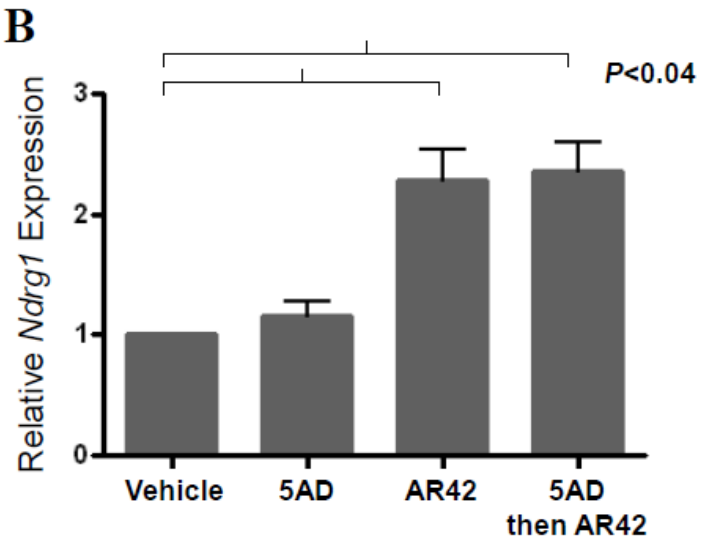


Figure 6 Epigenetic Modifiers Reduce Leukemic Burden in *Mll^{PTD},Flt3^{ITD}* Transplant: Leukemic mice were sacrificed 3/4 of the way through treatment to observe the effects of treatment with 5AD and/or AR42 on their total leukemic load. **A.** Peripheral blood smears show a marked reduction of blasts in treated mice compared to the vehicle treated controls (100x magnification). **B.** Spleen infiltration is common in our mouse model, and spleen samples from all treatment groups had significant weight reduction when compared to vehicle controls ($p<0.003$).





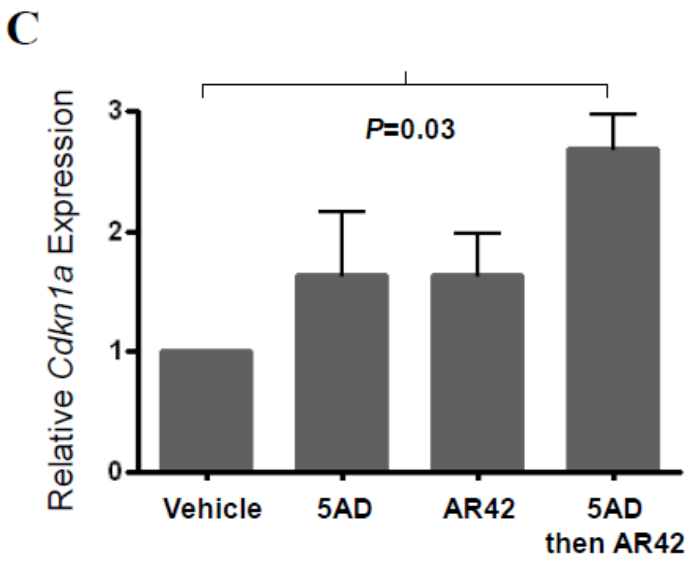
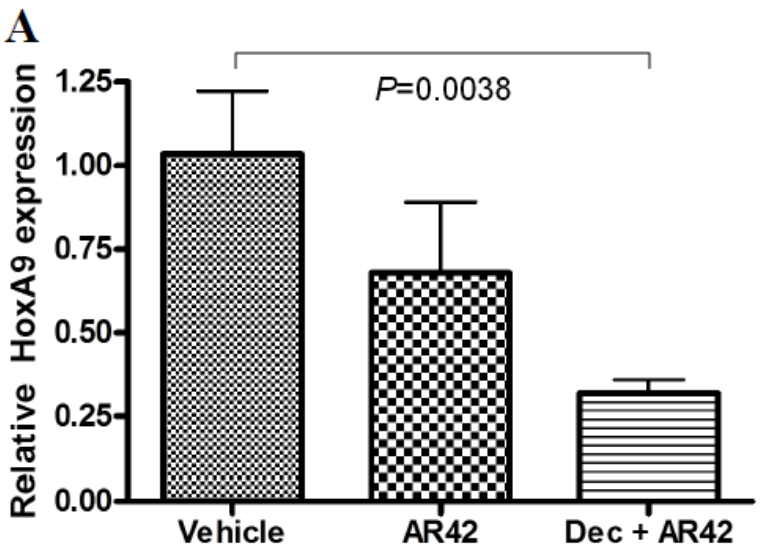


Figure 7 Epigenetic Modifiers Increase Tumor Suppression Expression in Primary *Mll*^{PTD},*Flt3*^{ITD} Mouse Cells: *Mll*^{PTD},*Flt3*^{ITD} whole bone marrow was cultured *ex vivo*. Cells were either treated with 5AD or vehicle, followed by AR42 or vehicle 24 hours later (cells were treated for a total of 48 hours). This yielded four different conditions: Vehicle, 5AD alone, AR42 alone, and 5AD & AR42. RNA was isolated, reversed transcribed to cDNA, and expression of tumor suppressors quantified via real-time RT-PCR. **A. & B.** AR42 alone was able to significantly increase the expression of *Rb* and *Ndrg1* as a single agent. **C.** The combination of 5AD and AR42 induced a significant increase in *Cdkn1a* compared to vehicle control where single drug treatment did not induce a significant increase in expression.


A
Relative HoxA9 expression
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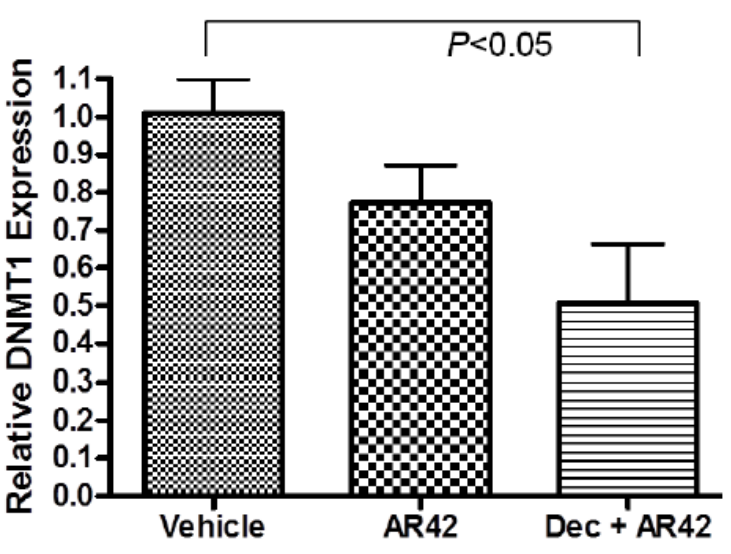
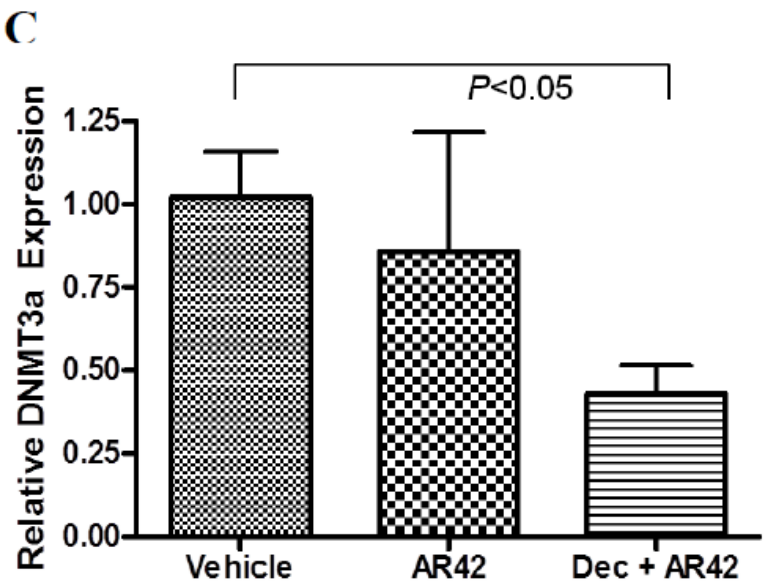
1.00
0.75
0.50
0.25
0.00
P=0.0038
Vehicle
AR42
Dec + AR42


B
Relative DNMT1 Expression
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P<0.05
Vehicle
AR42
Dec + AR42


C
Relative DNMT3a Expression
1.25
1.00
0.75
0.50
0.25
0.00
P<0.05
Vehicle
AR42
Dec + AR42

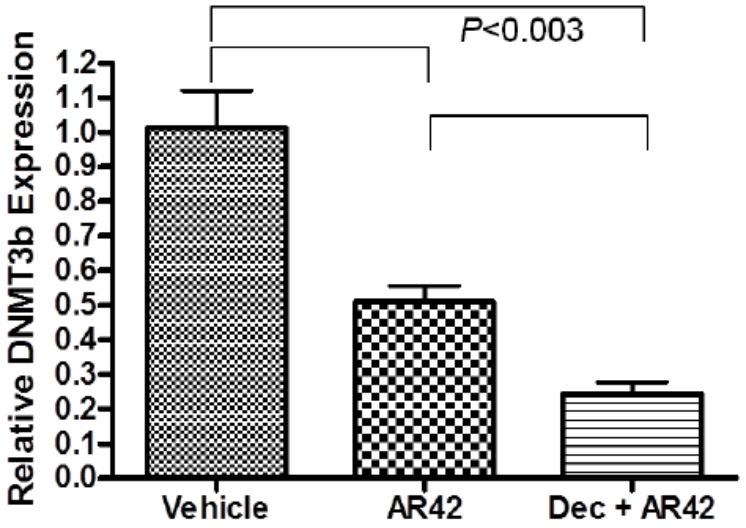

Relative DNMT3b Expression
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P<0.003
Vehicle
AR42
Dec + AR42

Figure 8 Epigenetic Modifiers Reduce Expression of Oncogenes in Primary *Mll*^{PTD},*Flt3*^{ITD} Mouse Cells : RNA was isolated from leukemic *Mll*^{PTD},*Flt3*^{ITD} whole bone marrow samples (collected after treatment *in vivo*), reverse transcribed into cDNA, and quantified by real time RT-PCR. **A.** Expression of *HoxA9*, a direct target of MLL, was significantly reduced leukemic when mice were treated with the combination of epigenetic modifiers. **B., C., D.** Similarly to results seen in *HoxA9*, *Dnmt1*, *3a*, and *3b* (all of which are upregulated in our mouse model) mRNA levels were significantly reduced after drug treatment.

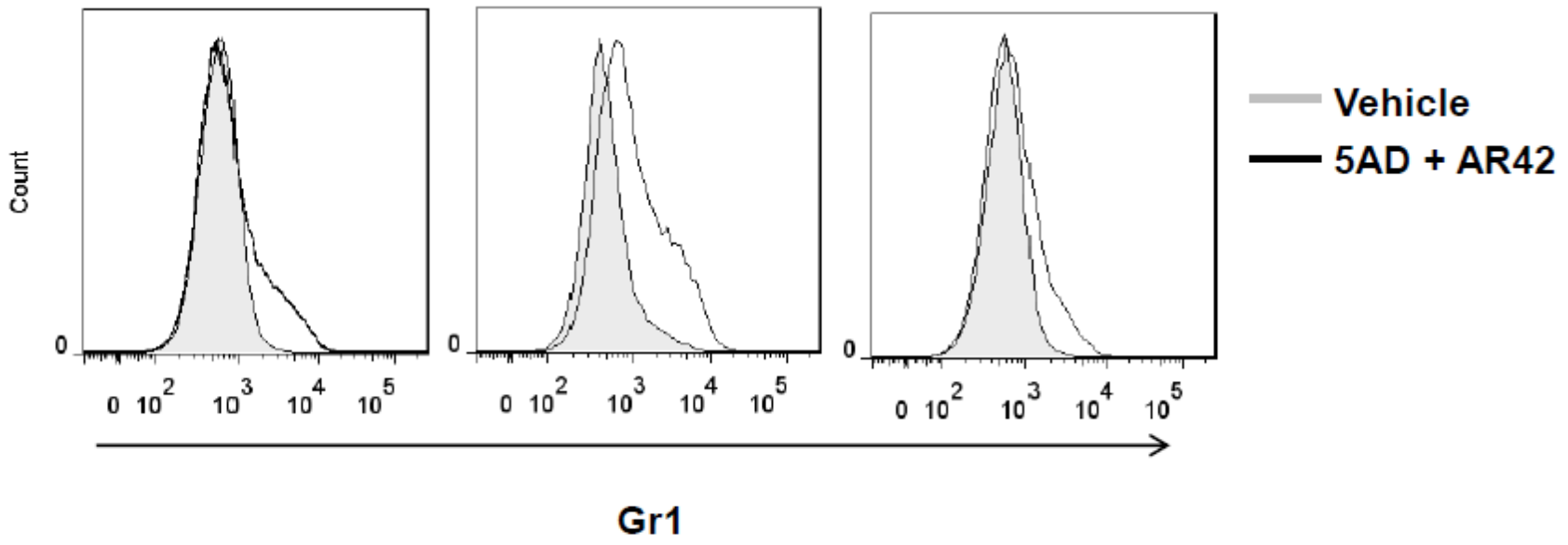
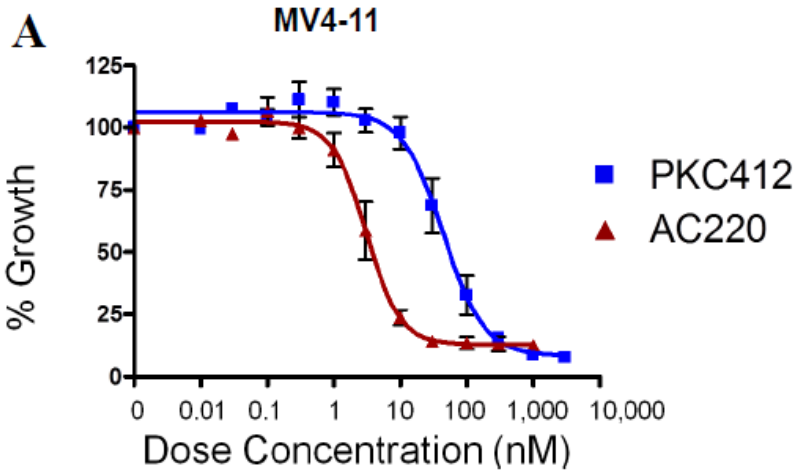
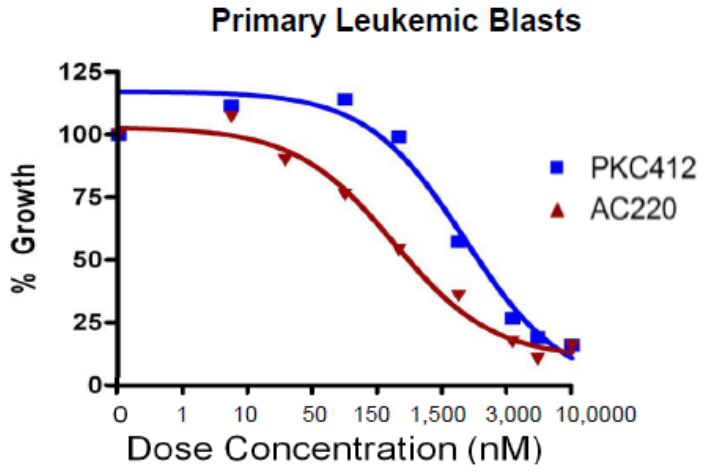


Figure 9 Epigenetic Modifiers Induce Differentiation in *Mll^{PTD},Flt3^{ITD}* Mice Treated *in vivo*: *Mll*^{PTD},*Flt3*^{ITD} whole bone marrow was obtained at sacrifice of mice 3/4 of the way through treatment with 5AD and AR42. Treated and control cells were stained for Gr1 (cell surface marker that is associated with myeloid cell differentiation) and measured via flow cytometry. Cells treated with a combination of epigenetic markers exhibited an increased level of Gr1 expression compared to a vehicle control sample.





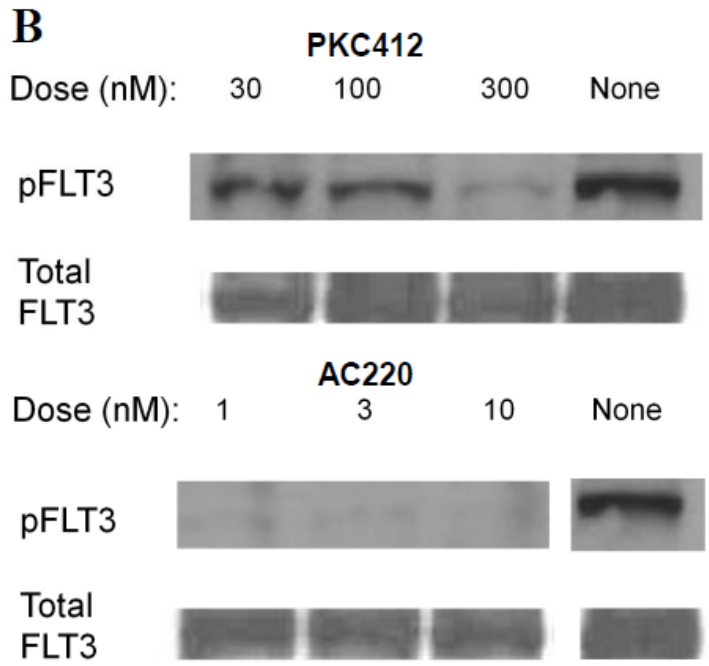
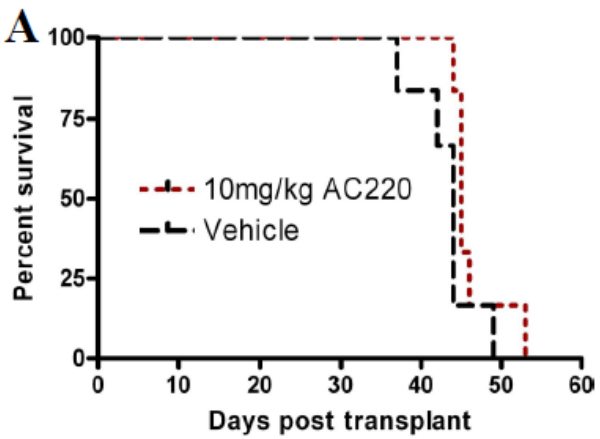


Figure 10 Tyrosine Kinase Inhibitors Effectively Target FLT3 Activation and Kill Leukemic Blasts: In order to test the efficacy of targeting $FLT3^{ITD}$ with receptor tyrosine kinase inhibitors, we tested both human cell lines *in vitro* and Mll^{PTD},$Flt3^{ITD}$ whole bone marrow *ex vivo*. **A.** As seen in MTS growth inhibition assays, AC220 displayed more potent growth inhibition on the MV4-11 human cell line than PKC412. **B.** Total Flt3 was isolated from whole cell lysates from treated MV4-11 cells via immunoprecipitation. pFLT3 levels were measured through western blot. AC220 was able to inhibit the phosphorylation of FLT3 at doses as low as 1 nM, while PKC412 only saw a minor effect at 300 nM. **C.** Growth inhibition of primary Mll^{PTD},$Flt3^{ITD}$ leukemic blasts was much less potent for either AC220 or PKC412. While AC220 remained more potent overall than PKC412, it required approximately 100x the dose to produce similar levels of inhibition seen in MV4-11.



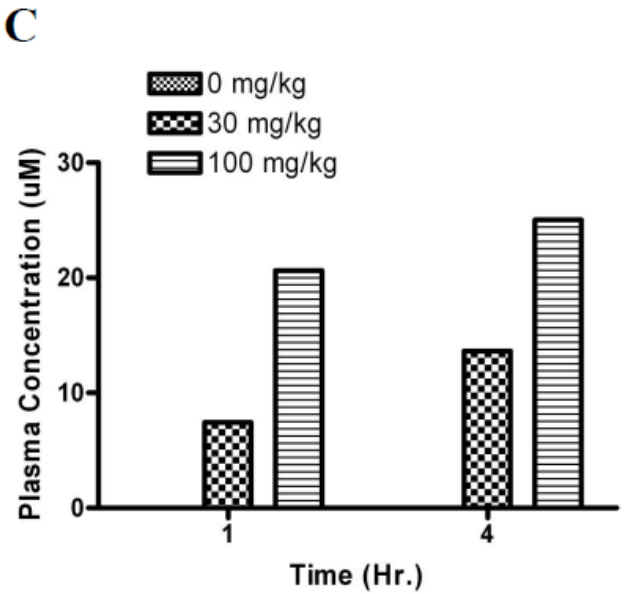


Figure 11 Low Dose of AC220 Unable to Increase Survival *in vivo*: Spleen cells from leukemic mice were transplanted into sublethally irradiated syngeneic mice. All AC220 doses were delivered to mice via oral gavage. **A.** Kaplan-Meier survival curve shows that a daily 10 mg/kg dose of AC220 for 28 days did not exhibit a significant increase in survival compared to the vehicle control. **B.** In order to determine if the downstream targets of Flt3 were being effectively silenced at this dose, a western blot of whole cell lysates (from flash frozen bone marrow and spleen cells) was probed for Stat5 phosphorylation. This *in vivo* dose of AC220 was unable to achieve the same level of less potent PCK412 on MV4-11 cells *in vitro*. **C.** A pharmacokinetic study was carried out in leukemic mice to better determine a dose that would reach an effective plasma concentration (measured by mass spectrometry) to match *ex vivo* killing levels for AC220 in primary mouse leukemic blasts.

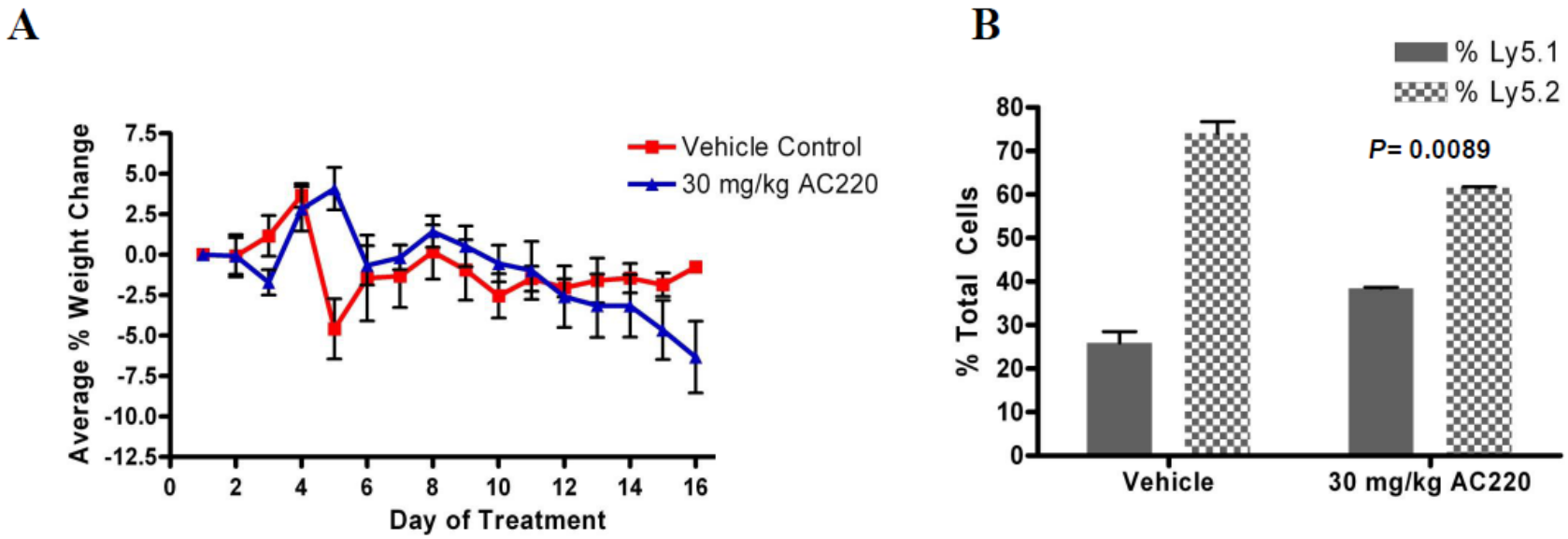
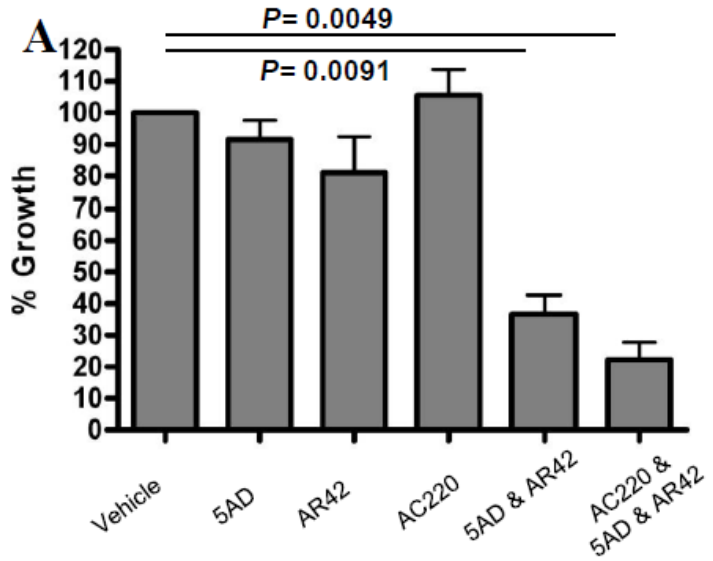
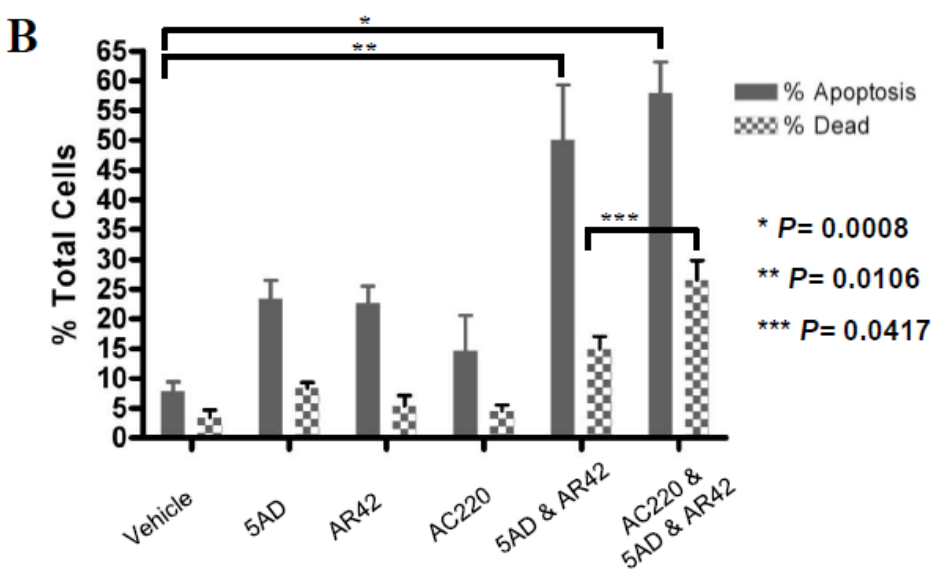
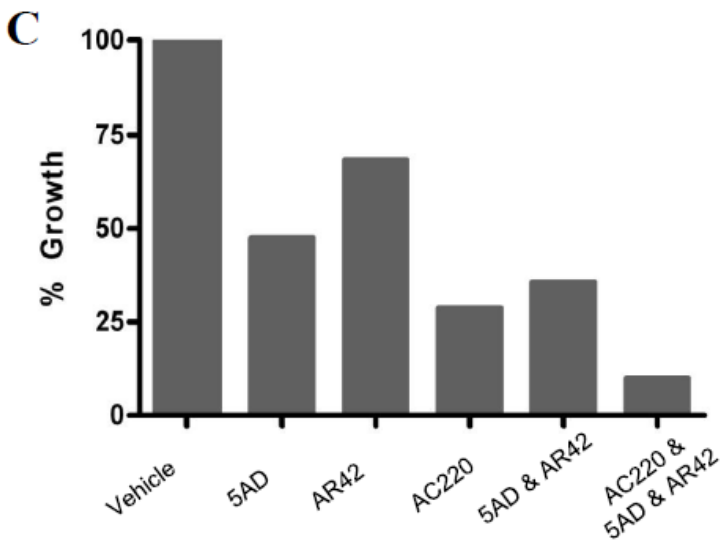


Figure 12 High Dose of AC220 Induces Toxicity *in vivo*:

Spleen cells from leukemic mice were transplanted into lethally irradiated syngeneic mice to provide a more uniform engraftment. After PK studies indicated that a dose of 30 mg/kg would be sufficient to reach plasma concentrations where AC220 had an effect *ex vivo*, a drug trial was performed *in vivo* to test the efficacy of this dose. **A.** The average percent weight change of treated and vehicle control mice was recorded throughout the trail. Initially, AC220 treated mice exhibited weight gain while the vehicle control did not, but after day 10, the treated mice began to quickly loose weight. **B.** On day 19, the remaining mice (one treated mouse was sacrificed on day 17) were sacrificed. Spleen and bone marrow samples were stained for Ly5.1 (wild type) and Ly5.2 (leukemic cells) cells and analyzed via flow cytometry. In AC220 samples, Ly5.1 cells were significantly higher and Ly5.2 were significantly lower, indicating treatment was able to target AML blasts.


A
P= 0.0049
P= 0.0091
% Growth
120
110
100
90
80
70
60
50
40
30
20
10
0
Vehicle
5AD
AR42
AC220
5AD & AR42
AC220 &
5AD & AR42


B
% Total Cells
65
60
55
50
45
40
35
30
25
20
15
10
5
0
*
**
***
% Apoptosis
% Dead
* P= 0.0008
** P= 0.0106
*** P= 0.0417
Vehicle
5AD
AR42
AC220
5AD & AR42
AC220 &
5AD & AR42


C
% Growth
100
75
50
25
0
Vehicle
5AD
AR42
AC220
5AD & AR42
AC220 &
5AD & AR42

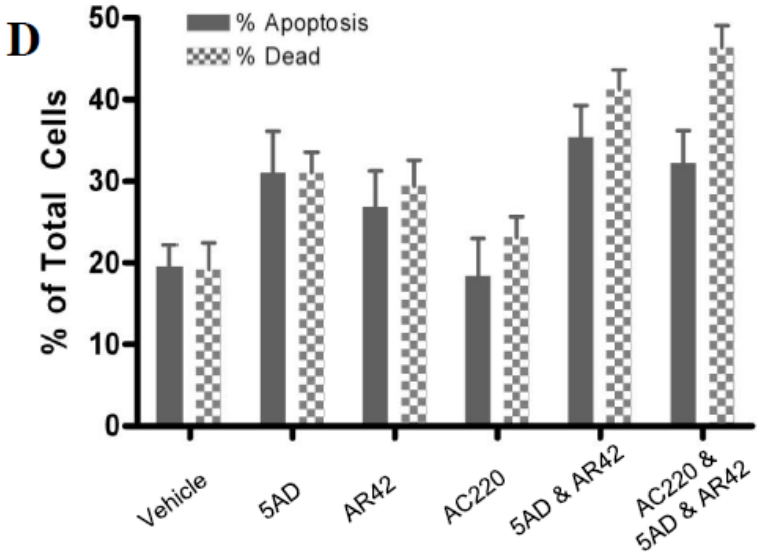

D
% of Total Cells
50
40
30
20
10
0
% Apoptosis
% Dead
Vehicle
5AD
AR42
AC220
5AD & AR42
AC220 &
5AD & AR42

Figure 13 Combination of Epigenetic Modifiers and AC220 Induce Growth Inhibition *in vitro* and *ex vivo*: Preliminary studies of combining 5AD, AR42, and AC220 were carried out on MV4-11 human AML cell lines *in vitro* and primary *Mll*^{PTD},*Flt3*^{ITD} leukemic blasts *ex vivo*. Cells were either treated with 5AD or vehicle, followed by AR42, AC220, AR42 & AC220, or vehicle 24 hours later (cells were treated for a total of 48 hours). This yielded six different conditions: Vehicle, 5AD alone, AR42 alone, AC220 alone, 5AD & AR42, and 5AD & AR42 & AC220. **A.** As measured by the MTS growth inhibition assay, MV4-11 cells treated with the combination saw the largest level of growth inhibition with all three drugs at 78%. Doses used: 5AD= 1,000 nM, AR42= 200 nM, and AC220= 3 nM. **B.** MV4-11 cells treated the same way were stained with Annexin V and 7-AAD and analyzed via flow cytometry. The triple combination saw approximately 58% of the total cells undergoing apoptosis. **C.** Growth inhibition of primary *Mll*^{PTD},*Flt3*^{ITD} leukemic blasts exhibited similar results in an MTS growth inhibition assay, with the triple combination producing the largest level of inhibition at 90%. Doses used: 5AD= 1,000 nM, AR42= 100 nM, and AC220= 1,000 nM. **D.** Finally, *Mll*^{PTD},*Flt3*^{ITD} leukemic blasts treated the same way were stained with Annexin V and 7-AAD and analyzed via flow cytometry. As in the human AML cell lines, the primary leukemic blasts exhibited an increase in apoptosis (32.9%).

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